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An Introduction to In Vitro Slice Approaches for the Study of Neuronal Circuitry

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Abstract

The acute slice preparation can be a powerful tool to study brain networks that would otherwise be difficult to manipulate at the synaptic and cellular levels. In the first part of this chapter, we discuss the specific challenges of preparing brain slices to study neural networks, and we review solutions to overcome problems that can be faced during slice preparation and maintenance. In addition, we describe slice preparations that preserve the connectivity between multiple brain areas, such as hippocampal and thalamocortical slices.

In the second part, we introduce several techniques that can be used to stimulate specific cells or networks in acute slices. We begin by reviewing methods for electrical stimulation, glutamate-based stimulation, and optogenetic stimulation. An additional procedure that combines the use of laser photostimulation with flavoprotein autofluorescence is also presented. We discuss advantages and disadvantages of these methods for neural network investigation in the acute slice preparation.

Key words: Slice preparation, Slice maintenance, Electrical stimulation, Laser photostimulation, Optogenetic stimulation, Flavoprotein autofluorescence

1. Introduction

Although brain slices were used since the 1930s to study brain metabolism (1), it was the extensive work of Henry McIlwain's group in the 1950s and 1960s that introduced the slice preparation as a reliable method for the study of brain physiology (2–4). Their work established an acute preparation with relatively well-preserved metabolism that offered a powerful environment for controlled manipulations. A critical breakthrough in the development of the technique was the observation of resting membrane potentials similar to those measured in in vivo preparations (5, 6). A further advance came with the first demonstration of simultaneous stimulation and recording, which opened the door to the study of neuronal circuitry in vitro (7).

More recently, a number of methods have been developed that maintain viable brain slices in culture for months (8, 9).

Such long-term organotypic cultures are beneficial in the study of the long-term effect of chemical compounds, ischemia, and the study of neurodegeneration and development. However, the acute slice preparation is more commonly used to study neuronal networks, and we focus on this method for the present review (for a review on organotypic slice cultures, see 10, for a protocol reference 11, and for applications 12).

2. Preparation of Brain Slices to Study Neuronal Circuitry

Several detailed descriptions of the instrumentation, as well as protocols for the preparation of brain slices, exist in the literature (13–19). We focus on the steps of slice preparation that are critical for neural network study.

2.1. Selection of Animal Model

The selection of an animal model can critically affect the outcome of slice physiology experiments. Rats and mice are commonly used since their small size permits the retention of a greater proportion of network connections using the typical slice thickness (300–400 μm). Cats, ferrets, and guinea pigs are other common choices, and relatively few primate slice experiments have been done (20–22). Young rodents (~1–2-weeks old) have often been used in the preparation of brain slices. However, older animals may be required for certain neuronal network studies. For example, it has been shown that spindle activity in the dorsal thalamus (which relies on the connectivity with the thalamic reticular nucleus) develops only after postnatal day 22 in ferrets (23). In addition, certain firing patterns, such as the repetitive bursting of some neocortical cells, are virtually absent in ferrets less than 3 months old (24) and in mice less than 1 month old (25). The synaptic response to repetitive activation of certain neocortical synapses in the rat has also been shown to change from depression to facilitation during the first postnatal month (26).

Brain removal for slice preparation in older animals, and, in general, in animals larger than young rodents (e.g., young cats), offers several challenges (18). The thicker skull slows down the removal of the brain, increasing the time between death and placement of the brain into artificial cerebrospinal fluid (aCSF) and, therefore, increasing the risk of hypoxic-ischemic injury. A second problem becomes apparent when the slices are under the microscope. Visibility through the slice is largely reduced due to the increased myelination density compared to juvenile animals. This makes it difficult to visualize cells and obtain whole-cell patch clamp recordings from healthy cells (commonly found in the depth of the slice). However, a number of steps can be taken during slice preparation to prevent hypoxia and improve cell visibility (see sections below).

2.2. Slice Preparation

The success or failure of a slice electrophysiology experiment is often determined at the brain removal stage, as it involves a brief period of ischemia which, if protracted, can render a tissue slice useless. Oxygen is commonly supplied to brain slices in the form of a gas mixture (95% O_2, 5% CO_2) bubbled into the aCSF, but irreversible hypoxic-ischemic damage can occur between the time of decapitation and the placement of slices into the oxygenated aCSF, as well as in the deeper portions of the slice if the superfusion rate and/or oxygenation level is not sufficient. Several strategies can prevent damage caused by lack of oxygen and the related toxicity. For example, when using larger species such as cats, the animal can be kept alive under deep anesthesia while the skull is being removed (16). With rodents, after deep anesthesia, the animal can be transcardially perfused with chilled, oxygenated aCSF (18). This is intended to cool down the brain, thus reducing metabolic demand as well as the release of glutamate and associated excitotoxicity. The perfusion also serves to clear blood from the vasculature, which can also help with cell visualization in the slice (18).

Another common method to limit tissue damage is to modify the aCSF used for sectioning and incubation of the slices (Table 1). Brain hypoxia is accompanied by release of glutamate that can cause neuronal death through several mechanisms (27). First, the entrance of sodium during cell depolarization mediated by glutamate may trigger multiple events potentially leading to cell death, such as the influx of chloride and water (causing cell swelling), a sodium-induced increase in cytosolic calcium, or a decrease in intracellular pH (28, 29). These risks can be mitigated by replacing sodium chloride with sucrose in the aCSF (18, 30). Another mechanism of glutamate toxicity depends on the influx of calcium into the cell through NMDA receptors (31), which leads to the activation of a number of degrading enzymes (27). In this respect, the use of ionotropic glutamate receptor blockers, such as ketamine and kynurenic acid (32–35), or general blockers of synaptic transmission (36) can help preserve the viability of cells in the slice.

Paradoxically, a hyperoxic environment, which can be toxic, can also develop in portions of the slice (37) due to the surplus of oxygen bubbled into the aCSF (95% O_2). Oxygen derivatives can interact with unsaturated fatty acids in cell membranes (lipid peroxidation) and affect synaptic transmission and plasticity (38, 39). Oxygen derivatives can also inhibit glutamate uptake transporters, thereby contributing to excitotoxicity (38). Thus, including antioxidants in the aCSF could be particularly important when preparing brain slices for neuronal circuitry analysis. A number of compounds can be added to the aCSF during slice preparation and incubation to prevent cellular damage by oxidative stress (Table 1). For example, ascorbate is an important antioxidant that is not synthesized in the brain, and is quickly lost during slice preparation (37, 40–43), and including ascorbate in the aCSF has

Table 1
aCSF modifications suggested to prevent hypoxic-ischemic and/or oxidative damage in the slice preparation

Substance	General effect	Potential problems	Use and references
Receptor and channel blockers (often glutamate receptor blockers)	Prevent excitotoxicity	Some blockers may decrease ATP levels (e.g., thiopental (152)) or be difficult to wash out (e.g., phenytoin (34))	*Ketamine*: 1 mM during slice preparation (34, 36), 25 μM in recording chamber (32) *D-APV*: 10–100 μM in recording chamber (32) *APH*: 200 μM during preparation (36) *Kynurenate*: 10 mM during preparation (153), 5–10 μM in recording chamber (32) *Barbiturates*: Thiopental: 400 μM in recording chamber (154), 600 μM in recording chamber (152, 155); pentobarbital: 10–100 μM in recording chamber (156) *Lidocaine*: 10–100 μM during slice preparation (34) *Tetrodotoxin*: 200 μg/l in recording chamber (157) *Phenytoin*: 100–200 mg/kg i.p. before decapitation, and then 10 μM during incubation (34)
Calcium: magnesium ratio	General blockade of synaptic transmission (prevents excitotoxicity). Preferred method when channel or receptor blockade is an issue (36)	Ratio should be carefully chosen to prevent effects on cell excitability (34, 158)	0 calcium: 10 mM magnesium during slice preparation (32, 34, 36)
Sucrose instead of sodium chloride	Reduces swelling and lysis	May enhance GABAergic transmission and affect network plasticity (e.g., LTP (159)); sucrose concentration should be chosen to preserve aCSF osmolarity (18)	252 mM during slice preparation (30)

(continued)

Table 1 (continued)

Substance	General effect	Potential problems	Use and references
Choline chloride instead of sodium chloride	Reduces swelling and lysis	None found	110 mM during slice preparation (160–162)
Ascorbate	Prevents oxidative damage, therefore also reducing excitotoxicity, swelling, and lysis	None found	0.4 mM preserves the in vivo level in slices kept at room temperature; at higher temperatures, higher concentrations are required (37) 0.4–1 mM during preparation and recording (18) 1.3 mM during preparation only (161)
Lactate	Helps support metabolism during recovery from hypoxia (163)	Still controversial as a substitute to glucose (see 164, 165)	3–6 mM during preparation and recording (163)
Pyruvate	Supports metabolism, prevents oxidative damage and excitotoxicity	It may be neuroprotective only in certain conditions (166)	2 mM (18) 5 mM during preparation and recording (98)

been found to prevent the formation of peroxides and decrease the swelling of brain slices (37, 41, 43).

On the other hand, Moyer and Brown (18) chose to modify the preparation technique, rather than the composition of the aCSF, to improve slice quality. They reported that keeping constant osmolarity across all aCSF solutions used throughout the experiment, as well as preincubating the slices in 36°C aCSF for 30 min, substantially increased the viability of the tissue prepared from aging or aged rats.

2.3. Blocking the Brain to Study Long-Range Connections

The correct blocking and positioning of the brain with respect to the cutting blade are critical in determining the success with which the circuit of interest is included in the slice. With short-range connections, the pre- and postsynaptic components of the circuit can often be included in slices sectioned in one of the anatomical planes (coronal, parasagittal, horizontal) at the standard thicknesses (~300–400 μm). Procedures for obtaining a variety of slice preparations that require sectioning in atypical planes are available in the literature (see references in subsequent paragraphs). A few approaches are reviewed in brief below.

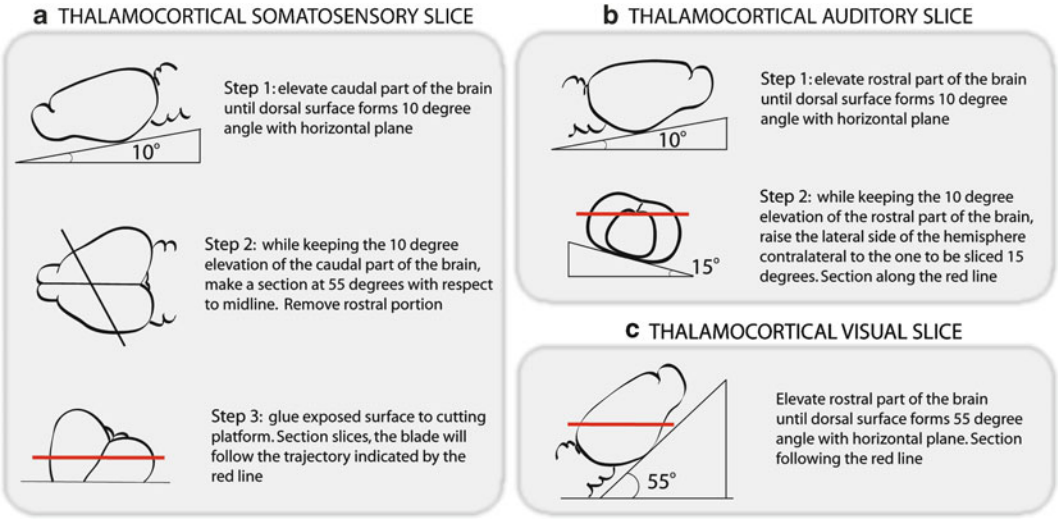

Fig. 1. Schematic representation of the position and blocking of a rodent brain to obtain sensory thalamocortical slices. *Red line* indicates the path that the blade follows when sectioning. (**a**) Somatosensory slice. (**b**) Auditory slice. (**c**) Visual slice. For initial description of these slice preparations, see references 52, 53, 55, respectively.

One of the most extensively used slices has been the hippocampal transverse slice, in which the sections are prepared by cutting close to perpendicular to the longitudinal hippocampal axis (i.e., parallel to the lamellae) to preserve the connections between all subregions of the hippocampal formation, from dentate gyrus to CA1 (14, 44–46). Due to the curvature of the hippocampus, a number of sectioning techniques have been developed. Cutting in a horizontal plane preserves the intrahippocampal connectivity in slices from intermediate to ventral hippocampus. A slight deviation from the horizontal plane allows the inclusion of parahippocampal regions in the hippocampus-entorhinal cortex slice (47–51). Dorsal hippocampus is best studied with sagittal or coronal sections. Alternately, the hippocampus can be dissected free before sectioning to produce slices from dorsal through ventral hippocampus (excepting the extreme septal and temporal poles).

Thalamocortical preparations (Fig. 1) have also become popular more recently, and a variety of methods have been developed to preserve connectivity in this complex network. The first approach, developed by Agmon and Connors (52), included the somatosensory thalamus and both feed-forward and feedback connections with somatosensory cortex. This was followed by slices including the auditory thalamocortical (and corticothalamic) pathway (53, 54), and slices including the visual thalamocortical pathway (55). In addition to sensory thalamocortical slices, preserved connectivity has also been demonstrated in slices between the midline thalamus (including the mediodorsal nucleus among others) and the anterior cingulate cortex (56). The potential of the thalamocortical slices goes beyond the study of the thalamocortical synapse because thalamocortical and

corticothalamic fibers travel across the thalamic reticular nucleus in their way to the cortex or the dorsal thalamus. Thus, some of these slices are also useful for studies of the network including the thalamic reticular nucleus (57, 58). Likewise, the thalamocortical preparation may also be used to study the intracortical spread of thalamic stimulation (59) as well as the specific and nonspecific thalamocortical projections (60, 61). Furthermore, thalamocortical preparations may include currently unexplored connections; such is the case of the auditory thalamocortical slice, which may also include thalamostriatal connections (62).

Within the neocortex itself, coronal, parasagittal, or modifications of these planes have been used to study cortical networks within and across functional regions. For example, coronal slices (63) or slightly off-coronal slices (64) include reciprocal connections between primary (V1) and secondary (lateromedial) visual cortex. In the somatosensory cortex, the plane of sectioning can be modified in order to preserve connectivity across particular barrels. For example, Finnerty et al. (65) cut slices at 45° with respect to the sagittal plane in order to obtain, in the same section, barrels receiving input from each of the whisker rows on the rat snout. Additional examples of slices that preserve both pre- and postsynaptic components include the mamillothalamic projection to the anterior dorsal thalamic nucleus (66) and the inferior colliculus projection to the medial geniculate body (67).

However, including both pre- and postsynaptic components of a circuit in their entirety is not always possible, and one alternative has been to target the axons of presynaptic components of the circuit under study while recording the postsynaptic cells. Examples of this strategy can be found in cerebellar slice (68), visual corticothalamic and retinogeniculate pathways (69), hippocampo-accumbens pathway (70), cortico-cortical between hemispheres (71, 72), and corticothalamic projections to the anterior dorsal nucleus (66).

2.4. Maintenance During Recording/Stimulation

The final step to study neural networks in the slice is to ensure that the slices maintain physiological properties during the experiment. Even when all the steps are taken to increase the likelihood of obtaining slices with normal physiology, it should be remembered that this is, nevertheless, an artificial preparation. Indeed, there have been reports of differences in the levels of metabolites, ion homeostasis, and respiration rates in the slice compared to the in vivo condition (73). It has been suggested that most of these changes can be explained by three observations (73): (1) mechanical cell damage during sectioning affects at least 50 μm on each side of the slice (because metabolic parameter estimations are commonly calculated per total slice volume or weight and a fraction of the slice volume contains dead cells, metabolic parameter estimations in the slice likely underestimate the in vivo values);

(2) hypoxic-ischemic damage during preparation; and (3) overall low spontaneous activity due to diminished neuromodulatory input, which has the secondary effect of reducing metabolic demand. Thus, taking measures to prevent damage from hypoxia (as described in previous sections) and recording from deeper parts of the slice will ensure that conditions more closely mimic the in vivo preparation. In addition, metabolic parameters (e.g., ATP levels) are the lowest right after preparation, and they recover to steady state in 2–4 h (14) with most of the recovery occurring in the first hour after slice preparation. Accordingly, incubation for at least 1 h before recording helps to ensure that the investigators are conducting their experiments at steady state. Once slices are in the recording chamber, superfusion rates can affect the oxygen level inside the tissue. Hypoxic conditions have been found at 150 μm of depth with superfusion rates of 3 ml/min or less (74). Therefore, superfusion rates of 3–6 ml/min in chambers with a total volume of 0.5 ml are recommended to provide appropriate oxygenation (75). Because the mechanical damage and level of hypoxia may vary from day to day causing variability in the data across recording sessions, care should be taken to standardize slice preparation across experiments.

2.5. Reliability of Cellular and Network Properties

Despite the substantial differences in the experimental conditions of neurons in the slice preparation compared to those found in vivo, several studies have suggested that many of the neuronal firing properties described in the slice are similar to those found in vivo provided that (1) the solution bathing the slice resembles in vivo CSF; (2) the temperature is kept within 5–10°C of the physiological temperature; and (3) the animal is old enough for the expected firing properties to have developed (24, 25, 76–81). Indeed, several types of characteristic firing patterns that have been described in neocortical cells (e.g., fast spiking, intrinsic bursting, etc.) have been found in slices as well (in vitro: 82–84; in vivo: 85). In addition, other specific firing properties, such as bistable states in thalamic neurons, or spontaneous irregular firing in striatal cholinergic cells have been observed both in vivo and in the slice (thalamus in vitro: 86–89; thalamus in vivo: 86, 90; striatum in vitro: 91; striatum in vivo: 92).

Network properties in the slice also appear to, in many cases, reproduce results found in vivo. For example, network rhythmic activity in the slice preparation has been reported in a variety of brain regions, including slow-wave oscillations in cortical slices (93), theta rhythm in hippocampal slices (94), and spindle oscillations in thalamic slices (95). Significantly, postsynaptic potentials recorded in vitro also resemble those recorded in vivo (45) and components of local field potentials elicited by pathway stimulation in slices are similar to those reported in vivo (14, 45, 96).

Maintenance of normal network physiology relies on maintenance of normal synaptic transmission. In addition to the parameters discussed in previous sections for obtaining slices with normal metabolic and cellular properties, attention should be paid to factors that may affect neurotransmission. For example, normal hippocampal network activity can be affected by oxygen levels; it has been shown that hippocampal sharp-wave ripples and fast oscillations are substantially reduced when perfusion rates are too low to provide normal oxygen levels (74). The concentration of the most common energy source used in aCSF, glucose, can also affect some connections (97). Further, the choice of energy source was found by Holmgren et al. (98) to dramatically affect the postsynaptic effects of GABA by altering E_{GABA} and the resting potential, leading the authors to propose the use of aCSF containing more physiological combinations of energy sources (such as pyruvate and ketones in addition to glucose).

Likewise, concerns have been raised regarding the scarcity of synaptic activity and the lack of physiological levels of modulatory neurotransmitters, which are not commonly used in aCSF (99, 100). The lack of synaptic activity can be problematic when studying synaptic plasticity because it is well-known that synaptic strength depends on previous spiking history (101). Furthermore, ongoing spiking activity contributes to gain control (102) and the reliability (103) of input–output relationships. Low spontaneous activity may be related to a number of factors, such as the selective elimination of excitatory inputs during sectioning. A possible solution to this problem would be to mimic the excitatory and inhibitory potentials a cell normally receives in vivo with electrical current injection (102, 103). Low oxygen levels caused by the use of low superfusion rates for the aCSF may also contribute to decreased activity. This is suggested by the recent finding of increased frequency of spontaneous EPSPs and IPSPs when the aCSF is perfused at high rates (75). The lack of physiological levels of modulatory neurotransmitters could contribute to the reduced synaptic activity observed in brain slices; modulatory neurotransmitters affect resting membrane potentials, as well as intrinsic response properties (104–106) and synaptic behavior (107). A variety of modulatory neurotransmitters (such as taurine, D-serine, or the previously discussed ascorbate) are present in the normal CSF, but these neurotransmitters are not routinely included in the artificial CSF used with brain slices. It has been suggested that the aCSF used for recording could be made more similar to the normal CSF by including small concentrations of those neuromodulators, as observed in vivo (75). Investigation of network properties in the slice typically requires the introduction of controlled input to the system. Below, we introduce three forms of stimulation that can be used in the slice: electrical stimulation, glutamate-based stimulation, and optogenetic stimulation.

3. Forms of Stimulation of Neural Activity in the Slice

3.1. Electrical Stimulation

Electrical stimulation offers the capacity to stimulate neurons with a virtually limitless variety of stimulation protocols using tools that can be as simple as a broken-tip glass electrode. The major liability of this technique is the lack of precision in the actual elements being stimulated. Despite this, electrical stimulation has found wide use due to its ease of application and flexibility.

The easiest and lowest-cost method to stimulate brain slices electrically is to use a broken-tip (tip size 2–10 μm) glass microelectrode filled with aCSF, with a reference electrode placed in the fluid portion of the tissue chamber. A point of caution with this approach is that the stimulation current path runs from the stimulation point to reference electrode, potentially stimulating neural structures in this pathway. Metal electrodes are typically made of tungsten and often constructed in a side-by-side or concentric bipolar configuration, which limits the spread of stimulation current. Bipolar electrodes permit lower stimulation strengths to be used (108), are less likely to cause electrolytic damage to neural tissue, and have been shown to produce lower stimulus artifacts than monopolar stimulation (109). In addition, bipolar electrodes allow the investigator to precisely control the orientation of stimulation, which may be important for activation of axons (108). However, we have found concentric bipolar electrodes to be too bulky to stimulate small structures in the slice. The primary disadvantage to such electrodes is the potential additional time and/or expense in their fabrication, compared to glass electrodes. Other approaches involve the use of arrays of electrodes, which allow arbitrary spatiotemporal patterns of stimuli to be applied to the slice (110–112).

Several studies have examined the sensitivity of different neuronal substructures to extracellular current injection. Nowak and Bullier concluded that electrical stimulation in the slice preparation activated axons, rather than cell bodies (113). They further demonstrated that inactivation of initial axonal segments via depolarization block had very little influence over stimulation threshold, suggesting that axonal branches, rather than initial segments, are responsible for triggering action potentials caused by extracellular current injection (114). It is also worth noting that manipulation of stimulation parameters or configurations can selectively activate different elements within a peripheral nerve; this was initially shown in vivo and recent work has suggested that these approaches may be used in the slice (115). A detailed description of these techniques is beyond the scope of this chapter, but the reader is referred to 116–120 for further reading.

Major issues to consider when developing an electrical stimulation paradigm are the efficacy of stimulation and damage to neural tissue. Most protocols call for trains of short-duration stimuli (e.g., <1 ms) since the efficacy of stimulation is a decreasing exponential function of stimulus duration (121), and long-duration stimuli do not permit the clearance of toxic metabolites that are generated during electrical stimulation. Most investigators use stimulation rates <50 pulses per second, as higher rates have been associated with depolarization block (122). Notably, depolarization block has been seen at 50 pulses per second with pulse durations of 100 μs, which yields a duty cycle of 0.5%. This suggests that the fundamental limiting factor is not related to persistent depolarization provided at high pulse rates. The actual waveforms of the individual pulses may also influence experimental outcome. For example, monophasic square-wave stimuli are commonly used because they offer high efficacy of axonal stimulation (121). In contrast, biphasic pulses are less likely to cause tissue damage, but have a lower efficacy of axonal stimulation. More complicated waveforms have been described for specific purposes, such as exponential waveforms to limit neuronal damage or quasi-trapezoidal/multistage waveforms for selective stimulation of subpopulations of neurons (121, 123, 124).

3.2. Glutamate-Based Stimulation

One liability of electrical stimulation in the slice is that electrical stimulation can trigger anti- and orthodromic activity in axons, dendrites, and cell bodies. This is often a hurdle that must be overcome by a series of controls to ensure that there is no stimulation of axons "passing through" the vicinity of stimulation. Glutamate-based stimulation avoids this problem since axons are not excitable by glutamate (125–127).

Glutamate-based stimulation is typically achieved via direct microapplication or laser-based photostimulation. Glutamate may be applied directly and locally via either microiontophoresis or pressure injection using a micropipette. Pressure injection involves the action of a high-speed valve to eject small volumes (generally, in the single-digit nanoliter range) of glutamate onto groups of neurons (121, 122). L-glutamate (1–2 mM) can be dissolved in aCSF and loaded into an injection pipette (1–5 μm tip diameter) and injection pressures of approximately 1–10 psi are typically used. The actual volume of glutamate ejected is proportional to the third power of the tip diameter. Therefore, if injection volume is important to a particular experimental question, each pipette should be individually calibrated (using a calibrated ocular to measure drop diameter) or a pipette fabrication approach that produces highly consistent tip size should be used. Microiontophoresis of glutamate is possible since glutamate carries a net negative charge at pH 8. Iontophoresis is generally done with pipettes (tip diameter 1–2 μm) with L-glutamate concentrations

of approximately 1 mM (123, 124). Higher concentrations (20–200 mM) may be required when smaller tip sizes are used (125, 126). Negative current pulses are used to iontophorese glutamate while positive current is used as a holding current between stimuli. One potential disadvantage to iontophoresis is the direct influence of tonic holding or injection currents on nearby neurons, and the relatively high glutamate concentrations necessary for this technique, which may produce receptor desensitization. While both approaches solve the fibers of passage problem, both techniques can be too cumbersome (compared to laser-based stimulation techniques; see below) for stimulation across an array of sites, and are therefore unsuitable for systematically mapping synaptic inputs. The main advantage of microapplication of glutamate via either iontophoresis or pressure injection is the relatively low cost and ease of setting up such a system.

One approach to enable multifocal stimulation with glutamate is to use scanning laser technology to focally activate glutamate. Toward this end, "caged" glutamate (L-glutamic acid α-(4,5-dimethoxy-2-nitrobenzyl) ester) was synthesized with a molecular cage that renders the glutamate molecule unable to bind to receptors (127). Exposure to UV light (355 nm) removes the cage enabling the glutamate to bind. Newer nitroindoline derivatives are more stable than the earlier generation molecules, retain rapid kinetics of photorelease, and are more commonly used for photostimulation (128). This technique yields high spatial resolution in two dimensions, with suprathreshold excitation being limited to an ~50–100-μm cylinder through the tissue sample (129). The speed of mapping, coupled with the relative ease of implementation with free software developed by Pologruto et al. (130), has allowed laser photostimulation to be extensively used to map synaptic inputs to specific areas in brain slices, both locally and across relatively long distances (131, 132).

One initial concern with laser-based photostimulation is that photostimulation of one group of neurons may cause a synaptically coupled neuron to spike ("synaptic driving"), which would confound the interpretation of presumed monosynaptic input maps generated using laser photostimulation. This issue was addressed by Katz and Dalva (133), who found that while recording extracellularly from cortical neurons spikes could only be elicited while stimulating over the cell body or proximal dendrites. Similar controls have been done by other investigators (129, 132), suggesting that synaptic driving in the cortical slice is unlikely.

One drawback to "traditional" single-photon-based photostimulation is the spread of activation caused by uncaging of glutamate molecules in the fluid along the light path and light scatter caused by the brain tissue outside of the plane of focus. This effectively limits the resolution of the technique such that it is unable to selectively stimulate individual neuronal substructures,

such as dendritic spines. An approach to dealing with this problem is to liberate caged glutamate using two-photon stimulation, where a high degree of spatial resolution can be achieved since only small volumes of tissue have the photon density to liberate glutamate. Two-photon uncaging is typically done with forms of caged glutamate with a large two-photon cross section (which is a measure of the probability of two-photon absorption), such as MNI-glutamate, and may be coupled with two-photon calcium imaging. As such, this technique is perfectly suited for visualization of calcium ion flux resulting from the activation of a single dendritic spine (134–136).

One note of caution regarding caged compounds is their potential to interact with receptors in the absence of photostimulation. For example, nitroindolinyl-caged GABA and nitroindolinyl-caged glutamate have been shown to interact with GABA receptors (128, 137), nitroindolinyl-caged glycine has been shown to interact with glycine receptors (128), and α-carboxy-2-nitrobenzyl-caged glutamate has been shown to interact with NMDA receptors (138). In the case of nitroindolinyl-caged glutamate, many investigators (including the authors) have used this compound without observation of seizure activity and with the ability to observe potent $GABA_A$ receptor activation (131). However, a new version of caged glutamate based on ruthenium chemistry (RuBi-glutamate) has been described which has a lower potential to cause GABA blockade. The high quantum efficiency of RuBi-glutamate allows lower concentrations of caged glutamate to be used, causing less GABA blockade (137).

3.3. Optogenetic Stimulation

An emerging technology for neuronal stimulation in the slice is the incorporation of genetically encoded light-sensitive channels into target neurons (for more details, see Part IV ("optogenetics") in this volume). The most commonly incorporated channel, channelrhodopsin2, is an algae-derived cation channel that opens in the presence of blue light (peak conductance at 470 nm). The reversal potential of the channelrhodopsin2 channel is approximately 0 mV; therefore, light-induced conductance in these channels depolarizes neurons. Channelrhodopsin constructs are typically combined with a fluorescent marker protein, such as yellow-fluorescent protein, to allow visualization of transfected neurons during experimentation. These constructs are generally delivered to target neurons via germline encoding, transfection via viral vector, or electroporation. The reader is referred to 138 and to the chapter by Cardin in the present volume for further description of approaches to channelrhodopsin2 gene delivery.

The main utility of "optogenetic" approaches is to achieve stimulation in a select population of neurons, targeted either genetically as belonging to a particular functional cell class (e.g., parvalbumin-containing interneurons (139)) by projection target (e.g., cortico-cortical neurons (140)) or other characteristics, such as cortical layer of origin (72). Once transfected, expression of

channelrhodopsin2 occurs throughout the cell, including dendrites and axon. The latter feature is particularly appealing for the study of long-range connections in the slice. When using electrical stimulation, the experimenter could be exciting an unknown, but potentially large, population of axons in addition to those of interest for the study. An improved approach would be to selectively transfect the projections of interest with channelrhodopsin2, cut slices of any desired orientation, and then selectively stimulate the axons using light of the appropriate wavelength. This strategy was successfully used to study somatosensory thalamocortical projections in the mouse (141).

Several technical issues are important to consider when comparing the utility of optogenetic approaches to more traditional approaches, such as electrical stimulation and glutamate uncaging. One issue is temporal resolution. Although the onset kinetics of channelrhodopsin2 are relatively fast and spike timing has high precision, there is a slow decay of activation lasting on the order of tens of milliseconds (142, 143). In addition, channelrhodopsin2 shows substantial desensitization after initial activation (143, 144). These factors limit the frequency with which action potentials can be driven in presynaptic axons. It should be noted that several new variations of the channelrhodopsin molecule have been engineered to have faster kinetics, and may be able to achieve more consistent high-frequency stimulation (145). An additional consideration is the compatibility of optogenetic approaches with other recording or imaging technologies. It is possible that direct illumination of a glass recording electrode may create an electrical artifact coinciding with the light pulse. Cardin et al. (146) described strategies to minimize these artifacts via use of a shorter wire or by coating the electrode with a nonreflective opaque substance (146) (for more details, see chapters by Siegle and Cardin in this volume). Coupling optogenetic stimulation with activation imaging is potentially complicated by the overlapping excitation spectra of traditional activation indicators and channelrhodopsin2. Such overlap would not permit selective stimulation and imaging to be separated in time (e.g., to stimulate with a pulse, then image over time) since the excitation light used for imaging would continue to cause stimulation. Newer red-shifted variants of channelrhodopsin (145) and/or imaging fluorophores (147) permit the flexible combination of optogenetic stimulation with fluorescence-based imaging (147, 148).

3.4. Laser Photostimulation Combined with Flavoprotein Autofluorescence as a Tool to Assess Connectivity in the Slice

A common problem faced by the slice physiologist is how to determine the degree of retained neural connectivity in a slice once it has been cut. For example, thalamocortical slices have been described for the study of the mouse somatosensory, auditory, and visual systems (52, 54, 55). Typically, only one slice (at best) per mouse has retained connectivity between thalamus and cortex. A rapid, noninvasive and sensitive method has been developed to assess directional connectivity in the slice using laser

photostimulation coupled to flavoprotein autofluorescence (LPFA). This approach allows the user to rapidly assess which slices are usable for the study of long-range connections. In addition, LPFA is a powerful tool that one can use to rapidly determine topographical maps of neuronal connectivity and to facilitate the development of novel slice preparations.

In LPFA, slices are bathed in caged glutamate, and a UV laser (355 nm) is used to focally uncage glutamate and orthodromically stimulate small groups of neurons in the slice. Flavoprotein autofluorescence (FA) imaging captures light produced in the projection field of the stimulated neurons. FA has recently been adapted for in vitro use in combination with a variety of stimulation methods (62, 66, 149). This technique relies on the green fluorescence (~520 nm) of endogenous mitochondrial flavin moieties, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) when they are exposed to blue light (~460 nm), and thus does not require dye loading. This avoids time-consuming equilibration with voltage- or calcium-sensitive dyes, which may alter the health of the slice and may produce heterogeneous uptake (150, 151). Under illumination at 460 nm, FMN and FAD increase their fluorescence by as much as 15–25% in response to neuronal stimulation, giving FA imaging one of the highest signal-to-noise ratios of the imaging methods employed to investigate network-level slice activity. Additionally, FA signal changes are sensitive to subthreshold postsynaptic activity and have a spatial resolution of approximately 100–200 μm (62).

Although FA can be coupled to electrical stimulation or local application of glutamate via pressure injection, we have found that photostimulation of glutamate combined with FA imaging offers the most rapid and noninvasive means to investigate slice connectivity. This combination has been used for rapid topographic mapping of connectivity within a slice, novel slice development (66), and connectivity assessment on a slice-by-slice basis (61). LPFA can be particularly useful when multiple structures are synaptically connected in a single slice, and can be used to identify connected loci for subsequent experimentation (Fig. 2). In consideration of practicality, adding FA imaging capability to an epifluorescence-equipped in vitro electrophysiology rig is straightforward. Beyond the appropriate filter set, the only additional materials are a moderate sensitivity camera and associated image acquisition software. The major drawback to this technique is the slow time course of activation, which is measured on the order of seconds. This potentially limits the ability to use FA to map the precise temporal evolution of a neuronal response, but is not a practical limitation when used for long-range spatial mapping. Thus, FA imaging offers a practical, cost-effective approach to rapidly assessing connectivity in vitro.

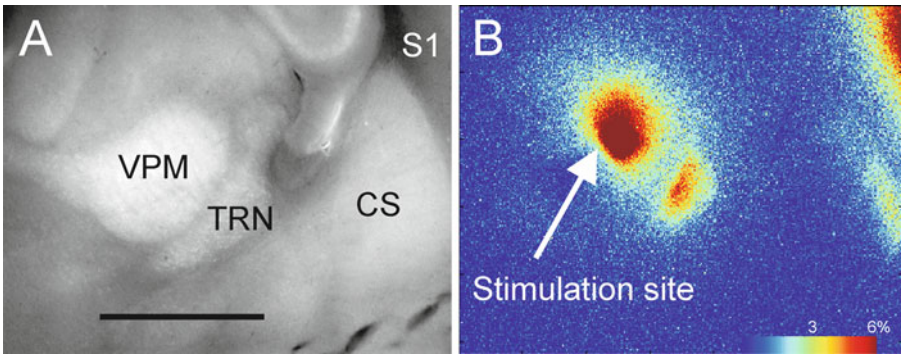

Fig. 2. Illustration of the use of LPFA to identify multiple connected areas in the mouse brain slice. (**a**) Bright-field image of the living slice at 5× magnification. *VPM* ventral posterior medial nucleus of the thalamus; *TRN* thalamic reticular nucleus; *CS* corpus striatum; *S1* primary somatosensory field. (**b**) $\delta f/f$ image of flavoprotein autofluorescence after laser photostimulation of caged glutamate in the VPM. Activation loci are seen in the TRN, CS, and the ventral portion of S1BF. Image is derived from a single trial consisting of a train of 20 pulses, 10-ms pulse duration, interpulse interval = 40 ms, pulse amplitude = 67 mW. Scale bar = 1 mm. For methodological details, see 62.

4. Summary

In summary, a wide spectrum of techniques is available for in vitro manipulation of neuronal circuitry. The remainder of this section focuses on implementation of some of the newest methodologies available to study neuronal networks in the slice preparation.

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